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Award Number: DAMD17-02-1-0488

TITLE: Analysis of Progestin Effects on Hepatocyte Growth Factor Signaling Pathways in Relation to Proliferation and Alveolar Morphogenesis of Normal Mammary Epithelial Cells in Vitro

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REPORT DATE: April 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual Summary (25 Mar 02 - 24 Mar 03)

4. TITLE AND SUBTITLE
Analysis of Progestin Effects on Hepatocyte Growth Factor Signaling Pathways in Relation to Proliferation and Alveolar Morphogenesis of Normal Mammary Epithelial Cells in Vitro

5. FUNDING NUMBERS
DAMD17-02-1-0488

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Progestin action in the normal adult mammary gland has a major role in the formation of the alveolar bud structure during development and pregnancy. We have determined that for P-induced proliferation and morphogenesis to occur, the presence of hepatocyte growth factor (HGF) is required (1). When added alone to mouse primary cultures from adult virgin mice, HGF induces proliferation and a ductal morphology. With the addition of P we observe increased proliferation and an alveolar-like morphogenesis similar to that seen in response to P in the in vivo gland. We have therefore proposed to study the effects of P on HGF/c-met signaling in an in vitro, serum free, three dimensional primary culture system using mammary epithelial cells. During the first year of this proposal methods for western blotting against c-Met were adapted for use in our culture system. The levels of c-Met were examined in response to the proposed treatments by immunostaining, showing that it is unlikely that the effects of HGF and progestins are mediated through changes in receptor expression. The effect of HGF and progestin treatment was also examined in pubertal mouse mammary epithelial organoids and did not exhibit the synergistic effect of HGF and progestin seen in adult mammary epithelial organoids.

14. SUBJECT TERMS:
breast cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Progestins (P) are major mitogens in the adult human breast and can significantly contribute to breast cancer risk. Progesterone action in the normal adult mammary gland has a major role in the formation of the alveolar bud structure during development and pregnancy. We have determined that for P-induced proliferation and morphogenesis to occur, the presence of hepatocyte growth factor (HGF) is required (1). HGF is growth factor that is produced only by the stromal cells of the mammary gland, and its cognate receptor, c-met, is expressed only in mammary epithelial cells. When added to mouse primary cultures alone, HGF induces proliferation and a tubulo-ductal morphogenesis. With the addition of P and HGF, we observe increased proliferation and an alveolar-like morphogenesis similar to that seen in response to P in the in vivo gland. For these reasons we believe HGF signaling through its receptor, c-met, activates signaling pathways that are affected by P resulting in increased epithelial cell proliferation and alveolar-like morphogenesis. We have therefore proposed to study the effects of P on HGF/c-met signaling in an in vitro, serum free, three dimensional primary culture system using mammary epithelial cells from adult virgin mice. We will examine the epithelium under conditions that induce growth and tubulogenesis (HGF alone) or conditions that induce growth and alveologenesis (HGF+P).

Body

Task 1: Assay receptor levels and activation in months 1-12

The first step in this task was to work out the methods in the mouse mammary cell line SP-1 that expresses high levels of c-Met (2). Figure 1 in the appendix shows western blot of c-Met of SP1 lysate (lane 1), an immunoprecipitation (IP) of c-met from the SP1

lysate (lane 2) and a blot for c-Met in the IP supernatant (lane 3) to show that c-Met was indeed immunoprecipitated. A protein standard ladder was used as a marker, to determine that the band detected was indeed c-Met (145kD). This figures confirms that our methods for detecting c-Met by western blot are indeed capable in cells known to be expressing the c-Met protein.

Our next effort in this task was to detect c-Met in our primary mouse mammary epithelial cells. This task is slightly more difficult due to the lower levels of c-Met expressed in primary cells compared to the SP1 cell line. Figure 2 shows a western blot of c-Met in three separate cultures of primary mouse epithelial cells that were maintained in media containing 5% FBS. Protein loading was equal between the different cultures to ensure that differences between the different cultures were not due to unequal protein loading. These results show that we are able to blot for c-Met in our primary mouse mammary epithelial cells.

The next task in the proposal was to determine the effects of hepatocyte growth factor (HGF) and progestins on the levels of c-Met. The original plan was to use western blot to examine the levels of c-Met under the conditions that lead to an alveolar-like morphogenesis (HGF + Progestin) or to a tubulogenic (HGF alone) response. However, we found an interesting article since the submission of this proposal which showed that luminal and myoepithelial show differential response to HGF treatment (3), so we therefore wanted to examine the effects of HGF and progestin on c-Met levels in both of these cell types. To do this, we used immunocytochemical staining of the mammary epithelial organoids in our three dimensional collagen gel culture system. Shown in figure 3A is an example of the staining of an organoid for c-Met (green) and the

myoepithelial cell marker, α-smooth muscle actin (red), followed by an overlay of the two stains. In order to insure that the staining for c-Met was specific a blocking peptide was used against the c-Met antibody. Figure 3B shows that the blocking peptide is able to block the c-Met staining and therefore indicates that the staining of the organoids for c-Met is specific. A time course experiment was then performed in order to examine the levels of c-Met in response to the treatments of basal media (BM), HGF, Progestin (R5020), and HGF + R5020 at 24, 48, and 72 hrs. The levels of c-Met were determined using a measure of relative fluorescence intensity. All images were captured under the same conditions in order to insure that changes in intensity were not due to factors other than changes in the levels of c-Met. Figure 4A shows the levels of c-Met in luminal epithelial cell at the three time points, and indicates that there are not any significant changes in the levels of c-Met due to the different treatments. Figure 4B shows the levels of c-Met in the myoepithelial cells at the three time points, and again there are not significant changes in the levels of c-Met as a result of the various treatments. Figure 4C is a comparison of the levels of c-Met in the luminal cells versus the myoepithelial cells. Under all treatments and at all time points it was observed that the myoepithelial cell expressed higher levels of c-Met than the luminal cells. This differential expression of c-Met supports the idea that examining both the two cell types may be necessary to determine how HGF and progestins function in the mammary gland. However, at this time all experiments proposed to be carried out as planned and any changes in the statement of work will be submitted for approval.

In addition to the work discussed above we have also investigated if the effects seen in response to HGF and progestins in the adult mammary epithelial organoids are

similar to those seen in pubertal mouse mammary epithelial organoids. Figure 5 shows a comparison of the response to treatments of HGF and progestin in the adult epithelial organoids versus the response of pubertal epithelial organoids. The morphology seen in the pubertal organoids is distinct from that of the adult; the alveolar-like response is not seen in the pubertal organoids in response to HGF + R5020 treatment. Figure 6 shows the proliferative responses of the pubertal and adult organoids to the treatments. Again, the pubertal glands show similar responses to the adult with the exception of the HGF + R5020 response; the increased proliferative response of HGF + R5020 treatment seen in the adult organoids is not seen in the pubertal organoids. We feel this observation provides us with an important tool to examine the downstream effectors of c-Met in both the pubertal and adult mammary epithelial organoids and determine how the response of the epithelial organoids to HGF and progestin occur. Differences between the adult and pubertal gland may help us to elucidate how HGF and progestins lead to increased epithelial cell proliferation and an alveolar-like morphology.

Currently, all experiments proposed are to be completed as planned. However, since approval of this proposal we have also been looking at the roles of Cyclins D1 and D2 in the formation of the alveolar-like response and it is something that we believe may play a role, and should therefore be studied further. There was some delay in completing all of the tasks planned in year one, which was due in part to the six month wait for animal use approval in order to begin many of these experiments.

Key research accomplishments

- Western Blot methods adapted from SP1 mouse mammary cell line for use in normal primary culture cells.
- c-Met expression levels examined using immunochemical staining techniques.
- Publication of the work that this proposal is based on.
- Response of pubertal mouse mammary epithelial cells to HGF and progestin examined

Reportable Outcomes

1. Since the submission of this proposal, the work on which it was based has been published in Endocrinology (1).

Conclusions

The first year of this proposal has yielded a promising start. Methods for western blotting against c-Met were adapted for use in our culture system. The levels of c-Met were examined in response to the proposed treatments by immunostaining, showing that it is unlikely that the effects of HGF and progestins are through changes in receptor expression. The effect of HGF and progestin treatment was also examined in pubertal mouse mammary epithelial organoids and did not exhibit the synergistic effect of HGF and progestin seen in adult mammary epithelial organoids. This provides us with a promising avenue to examine how progestins and HGF work together by comparing the adult and pubertal systems.

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1: Sunil N, B. J., Haslam SZ. (2002). "Hepatocyte growth factor is required for

progestin-induced epithelial cell proliferation and alveolar-like morphogenesis in serum-free culture of normal mammary epithelial cells." Endocrinology **143**(8): 2953-60.

2: Rahimi N, Tremblay E, McAdam L, Park M, Schwall R, Elliott B. (1996). "Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma." Cell Growth Differ. **7**(2): 263-70.

3: : Niranjan B, B. L., Yant J, Perusinghe N, Atherton A, Phippard D, Dale T, Gusterson B, Kamalati T (1995). "HGF/SF: a potent cytokine for mammary growth, morphogenesis and development." Development **121**(9): 2897-908.

Appendix

1.

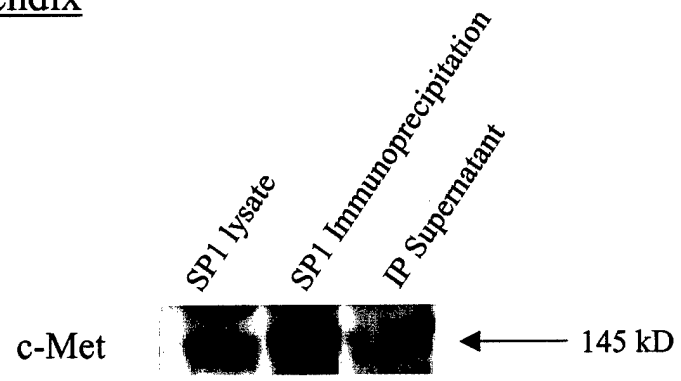

Figure 1. Immuno-blot and immunoprecipitation of c-Met from lysates of SP-1 mouse mammary cell line. 20μg of protein were added in each lane.

2.

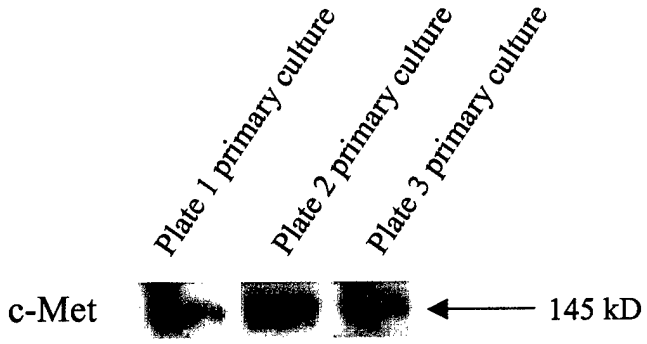

Figure 2. Immuno-blot of c-Met from lysates of primary culture mouse mammary cells. 30μg of protein were added per lane.

3.

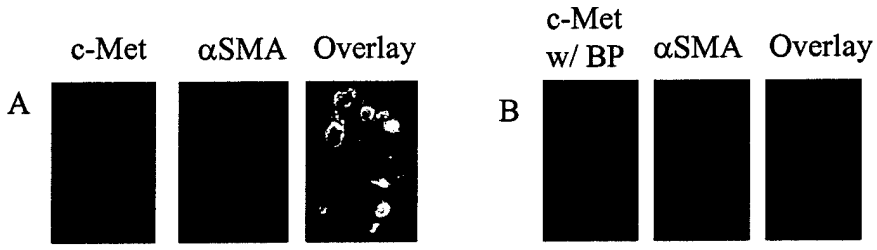

Figure 3. Immunocytochemical staining of c-Met in collagen gel cultures. A. Stain for c-Met is green, stain for myoepithelial cell marker, α-smooth muscle actin, is red, and areas stained yellow in the overlay show myoepithelial cells expressing c-Met. B. Staining for c-Met with a blocking peptide. Images all at 63x objective.

4.

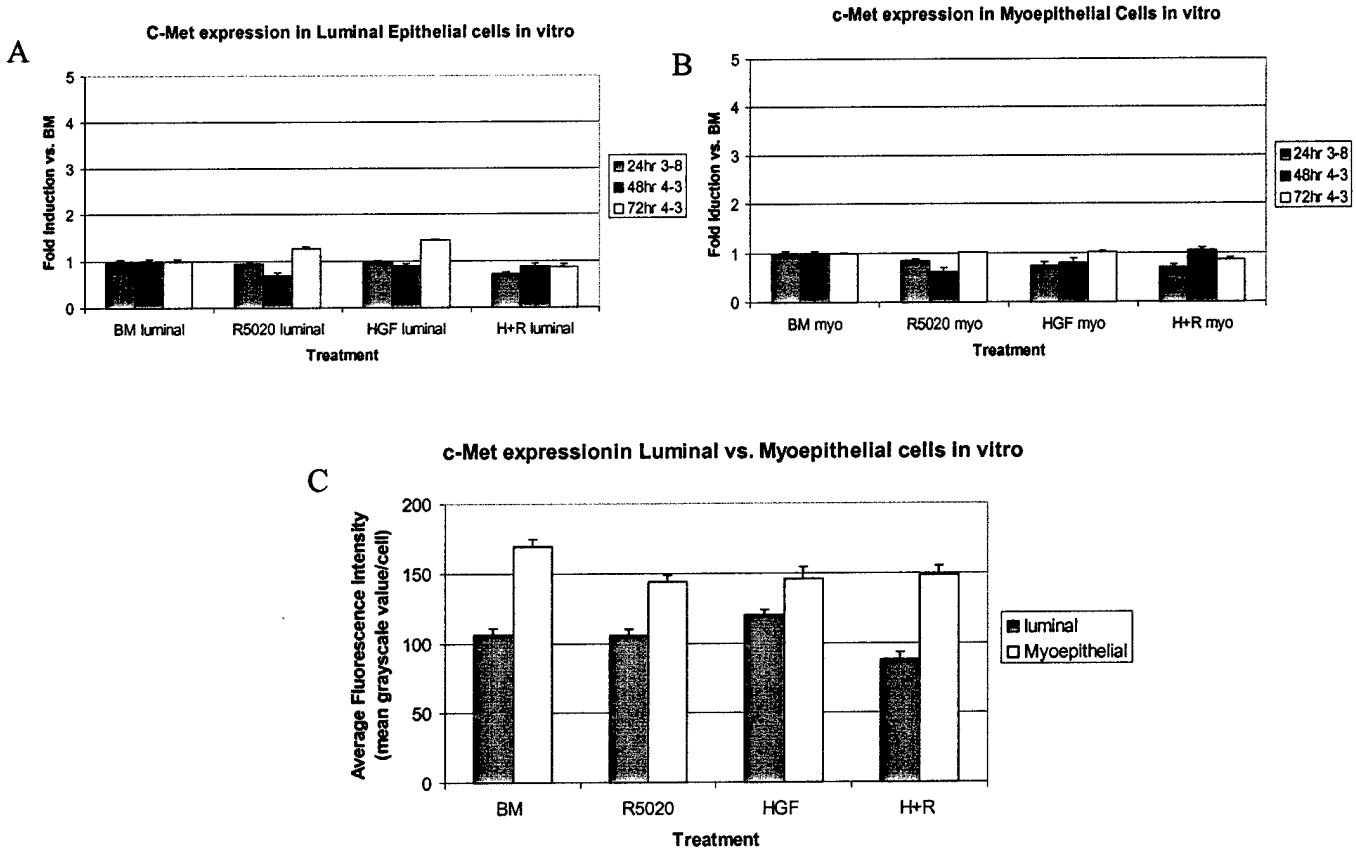

Figure 4. Effect of basal media (BM), progestin (R5020), hepatocyte growth factor (HGF), or HGF + progestin on c-Met expression in epithelial organoids cultured in collagen gels. Mammary epithelial cells in collagen gels were cultured alone (BM), in R5020 (10nM), in HGF (50ng/ml), or in HGF (50ng/ml) + R5020 (10nM). A. Expression of c-Met within luminal epithelial cells, B. Expression of c-Met within myoepithelial cells, and C. Expression of c-Met in luminal epithelial vs. myoepithelial cells. Each bar= M +/- S.E.M.

5.

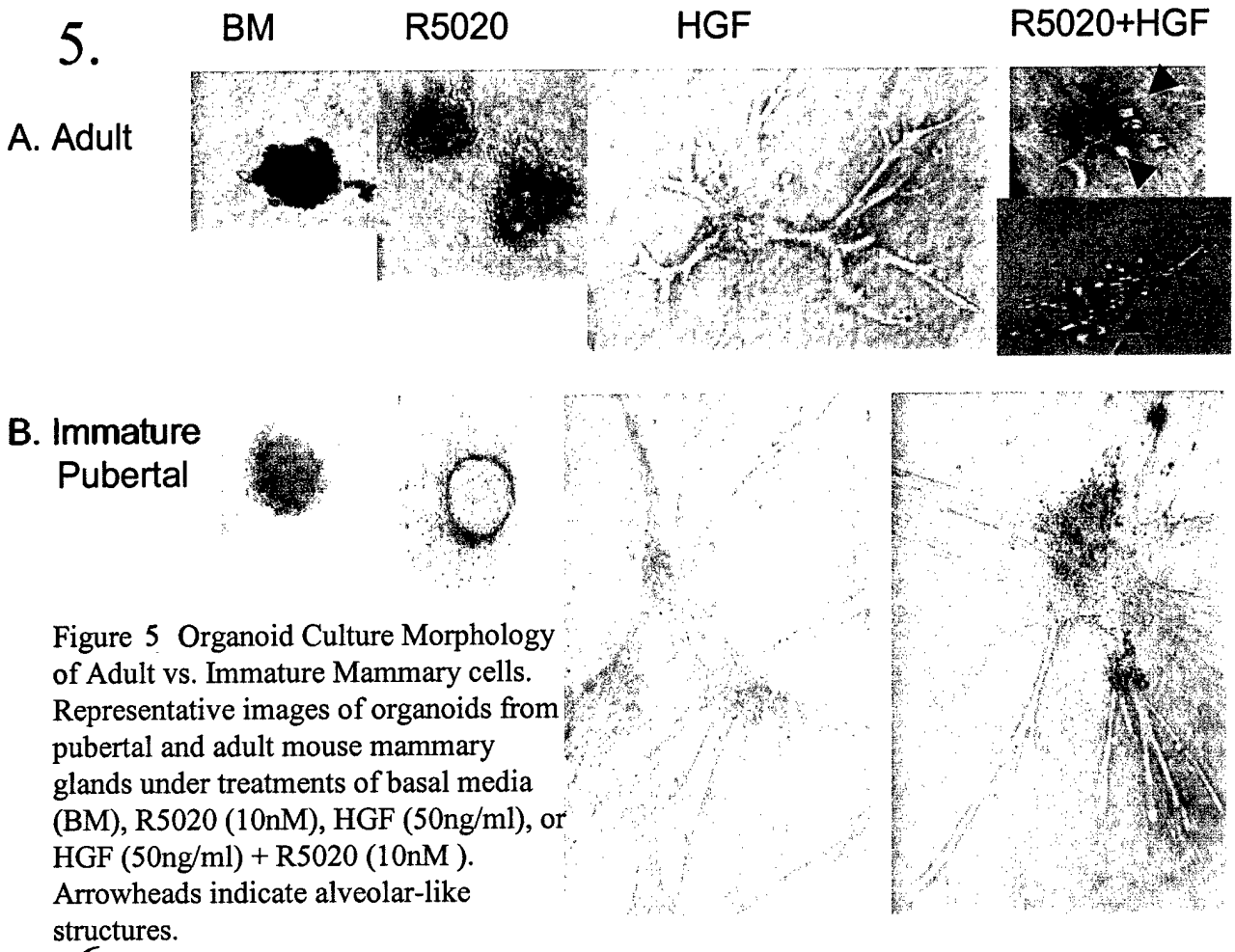

Figure 5 Organoid Culture Morphology of Adult vs. Immature Mammary cells. Representative images of organoids from pubertal and adult mouse mammary glands under treatments of basal media (BM), R5020 (10nM), HGF (50ng/ml), or HGF (50ng/ml) + R5020 (10nM). Arrowheads indicate alveolar-like structures.

6.

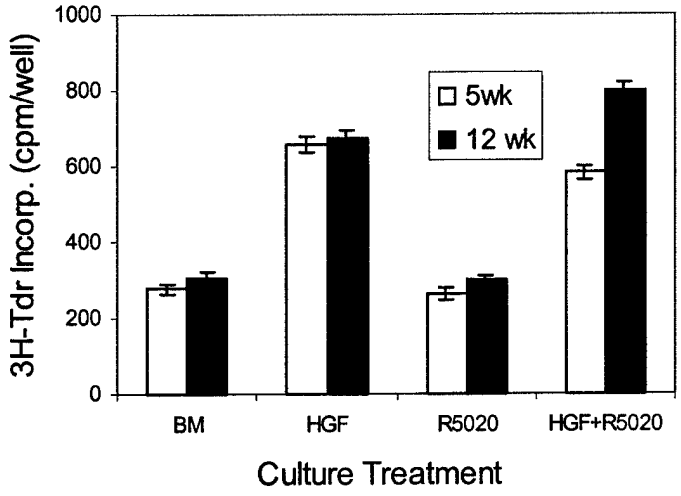

Figure 6 In vitro cell proliferation. Proliferation of adult or pubertal mouse mammary epithelial organoids as determined by 3H-Tdr incorporation under treatments of basal media (BM), R5020 (10nM), HGF (50ng/ml), or HGF (50ng/ml) + R5020 (10nM).